History of Advances in Genetic Engineering of Viruses Prior to COVID-19 Pandemic

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Benjamin Mueller and Carl Zimmer.
G.O.P. Senator's Report on Covid Origins Suggests Lab Leak,
but Offers Little New Evidence
(*The New York Times*, Oct 27, 2022).

Abstract. Due to the fact that to date, the question of the origin of SARS-CoV-2 has not been resolved yet, the author analyzed the main advances in the development of genetic engineering of viruses that took place before the onset of the COVID-19 pandemic.

The first artificial genetically modified viruses could appear in nature in the mid-1950s. The technique of nucleic acid hybridization was developed by the end-1960s. In the late 1970s, a method called the «reverse genetics» emerged to synthesize RNA and DNA molecules. In the early 1980-s, it became possible to combine the genes of different viruses and insert the genes of one virus into the genome of another virus. Since that time, the production of vector vaccines began. Currently, by modern technologies one can assemble any virus based on the nucleotide sequence available in the virus database or designed by a computer as a virtual model.

Scientists around the world are invited to answer the call of Neil Harrison and Jeffrey Sachs of Columbia University, for a thorough and independent investigation into the origin of SARS-CoV-2. Only a full understanding of the origin of the new virus can minimize the likelihood of a similar pandemic in the future.

Key words: reassortant virus; recombinant virus; chimeric virus; genetic engineering; reverse genetic; SARS-CoV-2; COVID-19

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1. Background

On December 31, 2019, the WHO's China Country Office was alerted to cases of pneumonia of unknown cause detected in Wuhan City, Hubei Province of China.[1]

2. Introduction

On January 21, 2020, Jordan Sather tweeted that the coronavirus that caused the epidemic in China was patented back in 2018.[2] Presumably he meant a patent describing an attenuated version of the coronavirus, which could be used as a vaccine for the treatment and prevention of coronavirus infection.[3] Sather also recalled that in early 2019, WHO named vaccine hesitancy as one of the top ten threats to global health. As it is known, *Ten threats to global health in 2019* was issued in the middle of January 2019,[4] and immediately caused an active discussion.[5-8] On September 12, 2019, WHO organized the Global Vaccine Summit, which discussed three important topics, namely: 'In Vaccines we trust', 'The Magic of Science' and 'Vaccines Protecting Everyone, Everywhere'.[9] Jordan Sather then asked questions whether the new disease was planned, whether it will be a way to raise money through the BigPharma system, whether the mass media is used to instill fear around a new disease, etc.[2]

Since that time, some media began to discuss the origin of the new virus, including the assumption of a virus leak from a bio-laboratory or even of a biological warfare.[10-13]

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On January 31, 2020 an article *Unique inserts in the 2019-nCoV spike protein*[14] was published followed by another one *Reduction and functional exhaustion of T-Cells.*[15] These discoveries demonstrated structural and functional similarities between two viruses and prompted a common-sense question about the origin of SARS-CoV-2.

On February 5, 2020, the VESTI.RU published a brief overview of the researches dealing with the achievements in genetic engineering of viruses over the past twenty years. Among them were: a mousepox virus constructed in Australia (2001); a poliovirus synthesized in the USA (2002); a «Spanish Flu» virus recovered and modified by the experts from the USA and Japan (2005-2008); an airborne avian influenza virus synthesized in the Netherlands (2011); a recombinant coronavirus, which poses an epidemic danger to humans, created by scientists from the USA, China and Switzerland (2015); a horsepox virus reconstructed by scientists from the USA and Canada (2018), etc.[16]

Confirmation of the possibility to obtain a new virus in the laboratory was the assembling of a synthetic coronavirus in a Swiss laboratory in February 2020. For the synthesis the scientists used a nucleotides sequence in the viral genome published by Chinese authors.[17] The discussion on the topic of the origin of the new virus that caused the COVID-19 pandemic resumed again.[18,19]

The version of the artificial origin of SARS-CoV-2 was supported by the Nobel laureates in Physiology or Medicine in 2003, Luc Montagnier, who was well acquainted with the achievements of the genetic engineering of viruses. According to Montagnier, the new virus was a side effect of research to develop a vaccine to prevent HIV infection.[20] Presumably, several genes important for the formation of immunity against HIV were inserted into the genome of the coronavirus. It only remained to weaken the virulence of a new virus and the vector vaccine against HIV infection would be ready.

Supporters of the natural origin of the new virus were in the majority. Among them, one can find many experts from all over the world who claimed that nowadays it was impossible to create a virus like SARS-CoV-2 in the laboratory. In March 2020, around 30 scientists published an article in the *Lancet*, which stated the following: «We stand together to strongly condemn conspiracy theories suggesting that COVID-19 does not have a natural origin. Scientists from multiple countries have published and analysed genomes of the causative agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and they overwhelmingly conclude that this coronavirus originated in wildlife».[21]

On April 18, 2020, Yuan Zhiming, a director of the Wuhan Institute of Virology, declared: «From my personal understanding of virology, there is no evidence to prove that the virus has artificial or synthetic traces. Besides, some scientists believe that to synthesize a virus requires extraordinary intelligence and work load. So, I have never believed that we humans would have the capability at this time to synthesize such a virus».[22]

A detailed and balanced analysis of the possible origins of the new virus was published in July 2020. In this study, Alejandro Souza concludes that «The various genetic peculiarities discovered in SARS-CoV-2 can be explained naturally. However, as the number of abnormalities causing some gain of function increases ... the statistical chances of such an event occurring decrease randomly in nature».[23]

Questions about the origins of the virus resurfaced in December 2020 when production of an Australian vaccine was discontinued as healthy vaccinated people became tested positive for HIV.[24]

The objective of this article is to study the main stages in the development of technologies used in the genetic engineering of viruses from the very beginning to the emergence of the COVID-19 pandemic.

3. The initial phase of research on the modification of the viruses

After the isolation of the tobacco mosaic virus (1935), the study of the structure of viruses, as well as the determination of the role of proteins and nucleic acids in the infectious process began.

Regular research into virus breakdown and reconstitution began in the mid-1950s. The book «Viruses» published in 1959 had a section devoted to the chemical basis of the infectivity of viruses. The chapter *Reconstitution of viruses from different strains* includes several topics, namely: Mixed viruses; Mixed nucleic acid viruses; Search for *in vitro*-produced mutants, etc. On the page 453 one can read the following: «Since it has become possible to demonstrate infectivity in degraded and reconstituted virus preparations, the aim has been to produce at will a new genetic (i.e., replicating) species of molecules».[25]

Viruses were exposed to either chemical or physical agents, which led to a change in their genotype and phenotype. An important aspect of such research was the isolation and study of parts of the viral genome that played a leading role in the process of infection and disease development.[26] Studies on the creation of pathogenic viruses using adaptive influence still continue in our time.[27].

4. Technologies used for nucleic acid synthesis and modification as well as nucleotide sequencing

The technique of nucleic acid hybridization was developed by the mid-1960s.[28] In the late 1960s, it became possible to insert DNA fragments of viruses into the DNA molecules of animal cells.[29] Then, a technology was developed to connect the ends of DNA molecules belonging to different viruses and bacteria; circular structures were constructed, consisting of the DNA of the simian virus 40 (SV40), the DNA segment of the lambda phage gene, and the DNA segment of the bacterium *Escherichia Coli*.[30] Approximately at the same time, experiments were carried out on the extracellular synthesis of nucleic acid

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molecules.[31] In 1976, a hybrid virus was constructed in which a segment of the DNA molecule of the lambda phage was inserted in place of the deleted part of the DNA of the SV40 genome.[32]

By 1978, a technique was developed to control the change in the genome of the virus, called the method of «reverse genetics». The RNA molecule to be replicated was used as a template, on which a DNA molecule was built with the help of the enzyme called *reverse transcriptase*. Then, the newly created DNA molecule was used as a template for constructing RNA molecules identical to the primary experimental molecule. At this stage of the synthesis, the enzyme *DNA dependent RNA polymerase* was used.[33]

In 2002, at the State University of New York, a template in the form of a DNA molecule was assembled from synthetic oligonucleotides, and then a full-sized infectious neurovirulent poliovirus was *de novo* synthesized; it was capable of paralyzing and killing mice. The nucleotide sequence of the viral genome was taken from a database available on the Internet, and the necessary synthetic oligonucleotides were purchased through a chemical sales network. This experiment proved the possibility of synthesizing an infectious pathogen by biochemical means *in vitro*, having only a description of the genome and synthetic nucleotides.[34]

In 2003, a report was made on a new technique that allows the rapid assembly of a synthetic DNA molecule with a size of 5 to 6 Kb. As confirmation of this, the complete infectious genome of bacteriophage X174, consisting of 5386 nucleotides, was assembled, for which chemically synthesized oligonucleotides were used. The infectivity of synthetic DNA was lower than that of a natural DNA, indicating approximately 10 errors per molecule.[35]

The creation of new bacterial and viral genomes was accompanied by the emergence of methods for determining the nucleotide sequence, i.e., sequencing of DNA and RNA molecules.[36-38]

5. Construction of reassortant viruses and production of vector vaccines

By the beginning of 1980s, genetic engineering had developed a technology that allows inserting a selected gene of one virus into a desired position in the genome of another virus, and then analyzing the phenotype of a new object. For example, one of the features of the epidemic rotavirus that causes diarrhea in humans is the difficulty of cultivation in tissue culture. After replacing several growth-limiting genes with cultured bovine rotavirus genes, a reassortant human rotavirus became capable of growing in tissue culture. This study was completed and a manuscript was sent to the editor of the Proceedings of the National Academy of Sciences of the United States of America on September 5, 1980.[39]

In 1982, the results of constructing and studying the characteristics of the new reassortant poxviruses were published. Dennis Panikali and Enzo Paoleti wrote: «We have constructed recombinant vaccinia viruses containing the thymidine kinase gene from herpes simplex virus. The gene was inserted into the genome of a variant of vaccinia virus that had undergone spontaneous deletion as well as into the 120-megadalton genome of the large prototypic vaccinia variant».[40]

Newly constructed reassortant viruses were now used for the production of a new type of vaccines. For example, Enzo Paoletti et al. published results of their study: «The technique involves translocating a particular gene from an infectious agent into the genetic material of the smallpox vaccine virus. This unique foreign gene, selected because it contains the information essential for the synthesis of an antigen important in immunity to that particular infectious disease agent, is now expressed under the regulation of the engineered smallpox vaccine virus. On immunization with this live recombinant vaccine, the body is fooled into thinking that it was infected by the foreign infectious disease agent and mounts a defensive attack resulting in immunity to that particular infectious agent». It is further reported that smallpox vaccine viruses were engineered to express genes encoding either the hepatitis B virus surface antigen (HBsAg), or the herpes simplex virus glycoprotein D (HSV-gD), or the haemagglutinin (HA) from influenza virus. This study was completed and published in September 1984.[41]

Vaccines made with application of a genetically modified non-infectious virus, into the genome of which genes taken from the infectious virus against which the preventive action is directed, were called «vector vaccines».[42]

Thus, any vector vaccine is the result of research on creation and production of a new genetically modified reassortant virus. One can assume that the construction of viruses similar to SARS-CoV-2 became possible around the border of 1980s and 1990s.

6. The rescue of the Spanish Flu virus that caused a pandemic in 1918-1920

In 1995, a team of experts from the Armed Forces Institute of Pathology (a U.S. government institution) began research to isolate the virus that caused the Spanish Flu pandemic. In 2005, scientists concluded that it was an avian non-reassortant virus that had adapted to humans.[43] The Spanish Flu virus was rescued by reverse genetics technique, and after the final manipulations, the deadly virus became human-specific.[44]

During the study of the certain parts of the Spanish Flu virus genome, specific gens were identified that could be responsible for high virulence and mortality. Then the construction of new reassortant viruses as well as testing their virulence began. In particular, recombinant viruses were generated in which the genes of the 1918 virus were replaced by genes from the modern human influenza virus H1N1, as well as recombinant viruses, in which the genes of the modern human influenza virus were replaced by genes of the 1918 virus.[45,46] It was assumed that understanding the virulence factors of future pandemic viruses would help to develop effective antiviral drugs that can stop a future pandemic.

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7. Continued work on the construction of the new reassortant viruses

Studies with potentially dangerous viruses included modification of genotype and phenotype among flaviviruses, poxviruses, orthomyxoviruses, coronaviruses, and others.

In 1999, scientists created a reassortant flavivirus in which the genes encoding two structural proteins of the Japanese encephalitis virus were inserted into the genome of the yellow fever virus. The new viruses grew in vertebrate or mosquito cells as well as their predecessors, although they did not share common mosquito vectors and reservoirs among vertebrates, and they differed in the clinical syndromes they caused.[47]

In 2001, a reassortant mousepox virus was constructed, into the genome of which the herpes simplex virus gene was inserted. In genetically resistant mice infected with the modified virus, there was an increase in the production of interleukin-4 and suppression of the cytolytic response of natural killers and cytotoxic T-lymphocytes. The fulminant mousepox with high mortality occurred even in the case of preliminary vaccination.[48]

Research on the creation of reassortant influenza viruses was not limited to work with the Spanish Flu only, but also spread to other strains. In particular, a 2008 publication states: «... we used reverse genetics to generate the 63 possible virus reassortants derived from H5N1 and H3N2 viruses, containing the H5N1 surface protein genes». Of the 63 reassortants, 13 posed the greatest threat to mammalian hosts. «... one of the most pathogenic reassortants contained avian PB1, resembling the 1957 and 1968 pandemic viruses».[49]

In September 2011, the 4th Conference of the European Scientific Working Group on Influenza (ESWI) was held in Malta, where a number of reports were presented describing the creation of genetically modified viruses with enhanced or weakened pathogenic functions.

One of the reports was titled: *Why is HPAI H5N1 virus not transmissible via aerosol? An extensive mutational and phenotypic analysis of mutant and reassortant H5N1 viruses*. The Methods section says: «We introduced several known adaptation mutations and exchanged several gene segments in an attempt to adapt HPAI H5N1 virus for efficient replication and possibly transmission in mammals».[50]

Another report of the conference was devoted to the construction *in vitro* of reassortant viruses resistant to the Oseltamivir (Tamiflu). They were obtained by coinfection of MDCK cells with influenza viruses belonged to the resistant and susceptible to the Oseltamivir strains.[51]

At the conference there were presented the results of animal experiments in which spontaneous mixing occurred between wild-type influenza viruses and live viruses of the attenuated strain used for the vaccine. As it turned out, the new reassortants were not more dangerous than wild-type parents.[52] It was also reported that a new reassortant virus was found in one of the patients; it included genes from seasonal and pandemic influenza viruses, but this natural reassortant did not pose a pandemic risk.[53] Based on the results of these studies, it can be assumed that the occurrence in nature of reassortant viruses that could cause a pandemic is not a common issue.

In a study sent to the *Nature* in June 2015, and published in November 2015, scientists from the United States, China and Switzerland explored the directions of possible mutations of the bat coronavirus, in which a relatively harmless virus would acquire new properties and be able to cause a pandemic in humans. Using the method of reverse genetics, a gene expressing the spike of the bat coronavirus SHC014 was introduced into the genome of the SARS-CoV virus. A new reassortant virus had the ability to effectively bind to angiotensin-converting enzyme 2 (ACE2), a receptor located on the cell membrane of various human tissues, as well as multiply in respiratory tract cells and achieve *in vitro* titers equivalent to epidemic strains of SARS-CoV. In addition, the resistance of the virus to the therapeutic and prophylactic drugs used to treat SARS was revealed. Based on their research, the authors expressed concern that the coronavirus could be the cause of a future pandemic.[54]

In 2018, Canadian and American scientists recreated the horsepox virus (HPXV). Ten large DNA fragments of 10-30 Kb each were synthesized based on the nucleotides sequence of HPXV, and then they were assembled together, removing excess sections. The synthesized virus was less virulent in mice than modern vaccinia virus, yet it provided vaccine protection against lethal infection with the natural virus.[55]

In October 2022, Da-Yuan Chen, et al. described construction of a recombinant SARS-CoV-2 in which the gene encoding the spike protein of the Omicron virus variant was inserted into the genome of the original SARS-CoV-2 virus. The new virus, in an experiment, caused severe disease in mice with a mortality rate of up to 80%.[56]

Thus, since the very beginning of the emergence of technologies that allowed constructing reassortant (recombinant, hybrid, chimeric) RNA and DNA molecules, study on creating new viruses which were not in nature have never stopped even during COVID-19 pandemic.

8. Restrictions on research leading to increased pathogenicity or transmissibility of the potential pandemic viruses

A number of the reports presented at the conference held in Malta in September 2011 caused a heated discussion among both scientists and journalists. On December 20, 2011, a spokesman for the National Science Advisory Board for Biosecurity (NSABB) stated that henceforth it is recommended that only the final results of experiments and conclusions be published, without a detailed description of the process used to create new dangerous viruses.[57]

On October 17, 2014, due to the growing threat of the emergence of new dangerous viruses in the environment, gain-of-function researches with the viruses in the United States were temporarily suspended.[58] However, on January 9, 2017, the moratorium was lifted. The commentary to the decree stated: «Adoption of these recommendations will satisfy the requirements for lifting the current moratorium on certain life sciences research that could enhance a pathogen's virulence and/or transmissibility to produce a potential pandemic pathogen».[59]

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9. Cases of leakage of dangerous pathogens from bio-laboratories

Despite numerous statements that research on virus modification were carried out in laboratories with a high degree of safety, nevertheless, cases of violations of the rules for storing and transporting dangerous viruses outside the laboratories are known. Here are just a few examples.

On August 5, 2019, the New York Times published an article titled: *Deadly Germ Research is Shut Down at Army Lab Over Safety Concerns*. The laboratory, based in Fort Detrick, Maryland, contained about 70 highly dangerous pathogens and toxins, including those that cause Ebola, smallpox, anthrax and plague, and the poison ricin. The reason for the closure of the laboratory was a problem with disposal of dangerous materials.[60] Research at the Fort Detrick lab was already suspended in 2009 due to the discovery of 9,220 vials of pathogens which were not listed in the database.[61]

Another dangerous case occurred in January-February 2009, when the Austrian pharmaceutical company Baxter sent vials of «vaccine» against influenza (H3N2) to laboratories in Germany, Slovenia and the Czech Republic. After the introduction of the «vaccine" to ferrets, some animals died. When checking, it turned out that the «vaccine» contained a live bird flu virus (H5N1). In the first explanation regarding the incident, Baxter representatives stated that the vaccines were contaminated with a dangerous virus by accident, probably during packaging. Later, a spokesman for Baxter admitted that instead of vaccines, they sent «experimental virus material», but it was not noted in the accompanying documents.[62,63]

In 2004-2005, the College of American Pathologists sent test kits to more than 3700 laboratories in 18 countries, including Belgium, Brazil, Canada, France, Germany, Israel, Italy, Japan, Mexico, Singapore, the USA, etc. While testing some of the samples an influenza A/H2N2 virus was identified. This influenza virus circulated in humans at the beginning of the pandemic in 1957-58, but employees of the laboratories were not informed about the possibility of the presence of a dangerous virus in the kits sent to test their qualifications.[64]

10. Conclusion

This review suggests that the emergence of new genetically modified viruses became possible no later than the mid-1950s. In the ongoing studies, natural viruses were treated with various physical or chemical agents, and then they were selected depending on the weakening or strengthening of the pathogenic functions of the virus. As is known, the first pandemic caused by a reassortant virus, including the genes of the avian and human influenza viruses (H2N2), occurred in 1957-1958.[65]

Later it became possible to combine the genes of different viruses and insert the genes of some viruses into the genomes of other viruses. Since that time, the production of vector vaccines has begun, for which genetically modified reassortant viruses were used. The construction of viruses similar to SARS-CoV-2 became possible around the border of 1980s and 1990s. It is likely that the first reassortant virus, which included the genes of four different virus at the same time, was discovered in nature during the «swine flu» pandemic that began in the spring of 2009.[66]

Currently, there are all the necessary technologies for assembling any full-sized virus based on the nucleotide sequence available in the virus database, or for creating a new artificial virus based on a virtual model offered by a computer.

The given above has confirmed that before the emergence of COVID-19 pandemic the ability of genetic engineering of the viruses was more advanced than needed to construct the virus which is similar to SARS-CoV-2. Thus, scientists around the world should support Neil Harrison and Jeffrey Sachs of Columbia University (USA) in calling for a thorough and independent investigation into the origin of SARS-CoV-2.[67] Only a full understanding of the origin of the new virus can minimize the likelihood of a similar pandemic in the future.[68]

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A list of the references includes some articles or manuscripts, which have not been peer reviewed yet, or were published and then retracted. They are not recommended to doctors who have not enough knowledge and experience to evaluate the quality of a study. Some of the web addresses were interrupted while the manuscript was being formatted. When looking for a source, restore an interrupted address

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